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(54) Title: ACETYLTRANSFERASE AND USES THEREOF

(57) Abstract: MCM3AP (mini chromosome maintenance 3 associated protein) is characterised herein as a nuclear localised acetyltransferase which acetylates both the chromatin-associated protein MCM3 (mini chromosome maintenance 3) and histone H3. The acetyltransferase activity is shown to repress DNA replication. Methods, uses and assays for obtaining agents useful in the modulation of cellular proliferation are provided.

ACETYLTRANSFERASE AND USES THEREOF

The present invention relates to screening methods, peptides, mimetics, and methods of use based on the surprising discovery and characterisation of an interaction between known proteins, and thus numerous cellular processes of interest in therapeutic contexts. The principal protein in question is MCM3AP which is shown to interact with histone H3 in addition to MCM3, it further being shown herein that MCM3AP acetylates MCM3 and histone H3 and that MCM3AP is localised in the nucleus in a MCM3 dependent manner. Prior to the making of the present invention, it was not known that MCM3AP had an acetyltransferase activity.

An essential feature of eukaryotic DNA replication is that DNA synthesis occurs once and only once per cell cycle. Although the detailed mechanisms that govern this regulation have not been clarified, several proteins are reported to be involved. In many eukaryotes, origin recognition complex (ORC) binds to replication origins in a sequence-specific manner [Bell, 1992][Bell, 1993][Micklem, 1993]. Cdc6 [Bell, 1995][Cocker, 1996][Leatherwood, 1996][Liang, 1995] and the MCM (mini chromosome maintenance) family consisting of six proteins (MCM2-7) [Romanowski, 1996][Chong, 1996][Kimura, 1995][Rowles, 1996][Todorov, 1995] is also implicated in the regulation of initiation of DNA replication. After mitosis, ORC and Cdc6 recruit MCM proteins to form a replication license in G1 phase, and DNA replication is initiated upon the activation of the complex by cyclin-Cdk and CDC7 kinases to start S phase [Jiang, 1999][Bousset, 1998][Donaldson, 1998]. Activation of the complex induces the binding of another protein, CDC45, to the replication origin. CDC45 is essential for recruitment of the DNA polymerase onto the origins [Kukimoto, 1999][Aparicio, 1999][Mimura, 1998].

The precise function of MCM proteins remains unclear. While a chromatin remodelling, helicase function has been suggested [Ishimi, 1997][You, 1999][Kelman, 1999], recent studies also suggest that the MCM proteins might possibly play a role in transcriptional activation. MCM5 interacts with the activation domain of Stat1a and overexpression of MCM5 stimulates the transcription initiated by Stat1a [Zhang, 1998]. Yankulov *et al.* reported a physical interaction between MCM proteins and general RNA polymerase II transcription machinery [Yankulov, 1999]. Hence MCM proteins may possibly participate in transcription of mRNA, in addition to DNA replication.

The DNA in the nucleus is wrapped around a histone-core which is a protein complex involving the four histones H4, H3, H2B and H2A. This DNA-histone structure (nucleosome) is not compatible with gene expression. Re-organisation of the nucleosome is required for transcription factors and RNA polymerase to have access to the DNA for transcription.

Histone acetyltransferases are implicated in the activation of RNA polymerase II-dependent transcription [Pineiro, 1991][Nightingale, 1998]. Although, the detailed mechanism by which acetylation of core histones influences transcriptional activation is uncertain, it is proposed that histone acetylation in a particular region of chromatin promotes destabilisation of histone-DNA interactions in the nucleosome, resulting in increased accessibility of the chromatin to the transcription machinery. The targeted histone acetylation of a particular region is achieved by recruitment of acetyltransferases to the responsive promoters. Some transcription cofactors, such as p300/CBP, PCAF, ACTR, SRC-1, Gcn5 and the TAF250 subunit of TFIID, are intrinsic histone

acetyltransferases and are recruited in a signal-dependent manner [Bannister, 1996][Ogryzko, 1996][Yang, 1996][Chen, 1997][Kuo, 1996][Mizzen, 1996].

Recent studies indicate that some histone acetyltransferases acetylate non-histone proteins and modify the function of their substrate proteins [Chen, 1999][Gu, 1997][Sartorelli, 1999]. The p300/CBP is an acetyltransferase which acetylates non-histone proteins p53 and ACTR, in addition to the core histones [Chen, 1999][Gu, 1997]. The acetylation of p53 by p300/CBP activates the DNA binding of p53. ACTR is one of the co-activators of the estrogen receptor and the acetylation of ACTR disrupts the association of ACTR with the promoter-bound estrogen receptor, triggering the cessation of hormone-induced transcription. Thus, protein acetylation like phosphorylation is one of the post-translational modifications that regulates protein function.

In a previous study, the present inventors isolated an MCM3 associated protein (Map80) by two-hybrid screening using MCM3 as bait [Takei, 1998]. The new data presented herein unambiguously demonstrates that the MCM3 associated protein (MCM3AP: database accession number AB 005544) is a novel acetyltransferase. Furthermore, MCM3AP is shown to be localized in the nucleus in an MCM3-dependent manner. MCM3 and histone H3 are also identified as substrates for this acetylation activity.

Based on the experimental work and discussion herein, the present invention in various aspects is concerned with the interaction of MCM3AP and substrates such as histone H3 and MCM3, the modulation of the acetyl transferase activity of MCM3AP and the modulation of the acetylation of histone H3 and MCM3AP by MCM3AP.

Various aspects of the present invention provide for the use of MCM3AP, MCM3 and histone H3 polypeptides in screening methods and assays for agents which modulate acetylation of MCM3 by MCM3AP and/or modulate interaction with and/or acetylation of histone H3 by MCM3AP, and agents which modulate the acetyl transferase activity of MCM3AP, thereby affecting a range of cellular processes in which MCM3AP is involved, including DNA replication.

Various further aspects of the present invention provide for the use of MCM3AP polypeptides in screening or searching for and/or obtaining/identifying a substrate which is acetylated by said MCM3AP. The inventors have identified two substrates (MCM3 or histone H3) which interact with and are acetylated by MCM3AP. Peptide fragments of MCM3 and histone H3 which modulate this interaction form the basis of aspects of the present invention. Further substrates may be identified and/or obtained using the screening and assay methods of the present invention.

Identification of key residues in MCM3 or histone H3 acetylated by MCM3AP may also be used in the design of peptide and non-peptidyl agents which modulate, particularly inhibit, acetylation of MCM3 or histone H3 by MCM3AP or other acetylase enzyme, as discussed further below.

Methods of obtaining agents able to modulate the acetylase activity of MCM3AP, the interaction of MCM3AP with histone H3 and the acetylation of MCM3 and/or histone H3 by MCM3AP include methods wherein a suitable end-point is used to assess interaction in the presence and absence of a test substance. Assay systems may be used to determine MCM3AP acetylase activity and/or MCM3AP interaction with histone H3 and/or

acetylation of histone H3 by MCM3AP and/or acetylation of MCM3 by MCM3AP or one or more other acetylases.

For acetylation assays, MCM3 polypeptides and histone H3 polypeptides may include full-length protein, a truncated portion or fragment, or a truncated portion fused to other protein (eg. GST), or a suitable variant, allele, mutant analogue or derivative of any of these.

Peptide acetylation assays may use peptides that comprise the acetylated regions of MCM3 or histone H3. The acetylation of any of the above may be assayed by any of a variety of procedures such as discussed below and may be adapted to high throughput screening approaches. Generally of most interest is modulation of the acetylation of histone H3 and/or MCM3 by MCM3AP. Detailed disclosure in this respect is included below.

Various methods and uses of modulators which inhibit or potentiate i.e. stimulate or increase interaction of MCM3AP and histone H3 and/or inhibit or potentiate i.e. stimulate or increase acetylation of MCM3 or histone H3 by MCM3AP and modulators that affect (i.e. reduce, inhibit, increase or stimulate) the acetylase activity of MCM3AP are provided as aspects of the present invention.

The purpose of disruption, interference with or modulation of interaction between MCM3AP and histone H3 or the acetylation of MCM3 or histone H3 by MCM3AP may be to modulate any activity mediated by virtue of such interaction, as discussed above and further below. Acetylation of MCM3 by one or more other acetylases may be modulated for the same or similar purposes.

In one general aspect, the present invention provides a method for identifying or obtaining an agent which modulates the acetylase activity of MCM3AP including:

(a) bringing an MCM3AP polypeptide into contact with a test agent; and

(b) determining acetylase activity of said MCM3AP polypeptide.

The present invention also provides a method for determining the acetylase activity of MCM3AP in the presence of a test sample including:

(a) bringing an MCM3AP polypeptide into contact with the test sample; and

(b) determining acetylase activity of said MCM3AP polypeptide.

An assay method for an agent which modulates interaction between MCM3AP and histone H3, may include:

(a) bringing into contact a first substance including a MCM3AP polypeptide as described herein, a second substance including histone H3 polypeptide as described herein, and a test compound; and,

(b) determining interaction between said first and said second substances.

A test compound or agent which reduces or inhibits interaction between MCM3AP and a substrate such as histone H3 may be identified and/or obtained under conditions in which, in the absence of the test compound being an inhibitor, the first and second substances interact.

A test compound or agent which modulates e.g. increases or

potentiates interaction between MCM3AP and a substrate such as histone H3 may be identified using conditions which, in the absence of a positively-testing agent, prevent or impair the substances interacting.

A test compound which disrupts, reduces, interferes with or wholly or partially abolishes interaction between said substances (e.g. including a MCM3AP polypeptide and including a histone H3 polypeptide), and which may thereby modulate MCM3AP activity, may thus be identified. Furthermore, agents which increase or potentiate interaction between the two substances may be identified using conditions which, in the absence of a positively-testing agent, prevent the substances interacting.

Another general aspect of the present invention provides an assay method for a substance able to interact with the relevant region of MCM3AP or histone H3 as the case may be, the method including:

(a) bringing into contact an MCM3AP polypeptide which interacts with histone H3, or a histone H3 polypeptide which interacts with MCM3AP as described herein, and a test compound; and

(b) determining interaction between said MCM3AP polypeptide or said histone H3 polypeptide and the test compound.

A test compound found to interact with the relevant portion of MCM3AP may be tested for ability to modulate, e.g. disrupt or interfere with, MCM3AP interaction with histone H3 and/or ability to affect histone H3 and/or MCM3AP activity or other activity mediated by MCM3AP as discussed already above.

Interaction and/or binding in the presence of a test substance may be compared with the interaction and/or binding of the MCM3AP polypeptide to MCM3 polypeptide or histone H3 polypeptide in comparable reaction medium and conditions in the absence of a test compound. A test compound able to modulate the interaction may be identified.

In preferred assays according to the present invention, the end-point of the assay, that is to say that which is determined in order to assess the effect of the test agent on the interaction of interest, is acetylation of histone H3 polypeptide or MCM3 polypeptide.

In a further general aspect, the present invention provides an assay method for an agent with ability to modulate, e.g. disrupt, interfere with, increases or potentiate acetylation of MCM3 and/or histone H3 by MCM3AP, the method including:

(a) bringing into contact a MCM3AP polypeptide, a test compound and a MCM3 polypeptide or histone H3 polypeptide; and,

(b) determining acetylation of said MCM3 polypeptide, or said histone H3 polypeptide.

An assay method may be performed under conditions in which, in the absence of the test compound being an inhibitor, MCM3AP will acetylate the MCM3 polypeptide or histone H3 polypeptide.

A test compound which disrupts, reduces, interferes with or wholly or partially abolishes acetylation of the MCM3 polypeptide or histone H3 polypeptide, and which may thereby modulate MCM3AP activity, may thus be identified and/or obtained.

A further aspect of the present invention provides an assay method including:

(a) bringing into contact a MCM3AP polypeptide which acetylates MCM3 or histone H3, a test compound and a MCM3 polypeptide or histone H3 polypeptide which includes a site acetylated by MCM3AP; and,

(b) determining acetylation at said site.

Of course, an MCM3AP, MCM3, or histone H3 polypeptide may include any suitable variant, analogue, mutant or derivative. Fragments of histone H3 or MCM3 may be employed which include any of the sites of acetylation, such as including one or more of the relevant lysine residues.

Acetylase activity may be determined by measuring the acetylation of one or more components of the test sample. Components acetylated in the assay are substrates of MCM3AP. Methods for determining MCM3AP acetylase activity in a test sample may include quantifying the amount of substrate in the sample.

Interaction, binding and acetylation may all be determined according to known methods described herein.

Agents which increase or potentiate acetylation of MCM3 and/or histone H3 by MCM3AP may be identified using conditions which, in the absence of a positively-testing agent, prevent acetylation. Such agents may be used to potentiate the function of MCM3, histone H3 or MCM3AP, and may have an effect for example on transcription and/or DNA replication.

In screening and assay methods of the present invention, the test compound or binding partner (i.e. MCM3 or histone H3 polypeptide) may brought into contact with MCM3AP polypeptide

in the presence of a suitable substrate such as acetyl Co-A.

Methods of determining the presence of, and optionally quantifying the amount of, a MCM3AP or substrate in a test sample may have a diagnostic purpose, e.g. in the diagnosis of any medical condition discussed herein (e.g. a proliferative disorder such as cancer) or in the evaluation of a therapy to treat such a condition.

As described above, it is not necessary to use the entire proteins for assays of the invention which test for binding between two molecules. Fragments may be generated and used in any suitable way known to those of skill in the art. Suitable ways of generating fragments include, but are not limited to, recombinant expression of a fragment from encoding DNA. Such fragments may be generated by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Small fragments (e.g. up to about 20 or 30 amino acids) may also be generated using peptide synthesis methods which are well known in the art.

In addition to interacting at the site of acetylation of MCM3 or histone H3, MCM3AP may interact with MCM3 and histone H3 at one or more other sites. Affecting interaction at such a site may have an effect on acetylation of MCM3 and histone H3 by MCM3AP. Polypeptides which include various fragments and derivatives of the MCM3AP, histone H3 and MCM3 proteins, may be used to analyse this, using techniques such as alanine scanning and deletion analysis.

Affecting the interaction of MCM3AP with acetyl Co-A may also have an effect on the acetylation of MCM3 and histone H3 by MCM3AP. The present invention also encompasses modulation of the interaction between MCM3AP and acetyl Co-A at any site which results in modulation of MCM3 and/or histone H3 acetylation. The interaction between MCM3AP and acetyl Co-A may be modulated at a site located between amino acid residues 370 to 532 of the MCM3AP polypeptide.

The present invention also encompasses modulation of interaction between MCM3AP and histone H3 at any site, preferably resulting in modulation of histone H3 acetylation.

The present invention also encompasses modulation of the interaction between MCM3AP and MCM3 at any site which results in modulation of MCM3 acetylation.

In any assay method according to the invention, the amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.001 nM to 1mM or more concentrations of putative inhibitor compound may be used, for example from 0.01 nM to 100μM, e.g. 0.1 to 50 μM, such as about 10 μM. Greater concentrations may be used when a peptide is the test substance. Even a molecule which has a weak effect may be a useful lead compound for further investigation and development.

A screening or assay method may include purifying and/or isolating a test compound and/or substance of interest from a mixture or extract, i.e. reducing the content of at least one component of the mixture or extract, e.g. a component with

which the test substance is naturally associated. The screening or assay method may include determining the ability of one or more fractions of a test mixture or extract to bind to MCM3 or histone H3 or determining the ability of one or more fractions of a test mixture or extract to bind to MCM3AP.

The purifying and/or isolation may employ any method known to those skilled in the art.

A MCM3AP, MCM3, or histone H3 polypeptide suitable for use in the assay and screening methods described herein may be a full-length histone H3 protein from a eukaryotic cell, such as a yeast or a mammal, for example a human. A suitable polypeptide may also be an analogue, derivative, allele, variant, such as a splice variant or mutant of the wild type protein sequence. The term also includes fragments of such sequences.

A suitable fragment of MCM3 or histone H3 may comprise a lysine residue which is the site of acetylation. A suitable MCM3AP polypeptide retains the acetylase activity of the wild-type MCM3AP protein. For example, such a polypeptide may comprise or consist of amino acid residues 370-532 of the MCM3AP sequence. Other suitable polypeptides may comprise or consist of amino acid sequence from the N or C terminal of MCM3AP.

A polypeptide which is an amino acid sequence variant, allele, derivative or mutant of an amino acid sequence described herein and may comprise an amino acid sequence which shares greater than about 60% sequence identity with the sequence shown, greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 70% similarity, greater than about

80% similarity, greater than about 90% similarity or greater than about 95% similarity with the amino acid sequence described herein.

Sequence comparison may be made over the full-length of the relevant sequence shown herein, or may more preferably be over a contiguous sequence of about or greater than about 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 233, 267, 300, 333, 400, 500, 600, 700, 720 or 750 or more amino acids, compared with the relevant amino acid sequence.

For amino acid "homology", this may be understood to be similarity (according to the established principles of amino acid similarity, e.g. as determined using the algorithm GAP (as described below) or identity.

Amino acid similarity is generally defined with reference to the algorithm GAP (Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul *et al.* (1990) *J. Mol. Biol.* **215**: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* **85**: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol.* ***147***: 195-197), generally employing default parameters.

The precise format of any of the screening or assay methods of the present invention may be varied by those of skill in the art using routine skill and knowledge. The skilled person is well aware of the need to employ appropriate control

experiments.

Compounds which may be screened may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants, microbes or other organisms, which contain several characterised or uncharacterised components may also be used.

It is worth noting that combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate an interaction. Such libraries and their use are known in the art, for all manner of natural products, small molecules and peptides, among others. The use of peptide libraries may be preferred in certain circumstances.

In various aspects, the present invention provides a modulator identified by a screening method of the invention, e.g. a substance which interferes with or interrupts, increases or potentiates interaction and/or binding of MCM3AP to histone H3. A modulator may reduce, interfere with or interrupt, increase, stimulate or potentiate acetylation of MCM3 or histone H3 by MCM3AP. Furthermore, a modulator may interfere with or interrupt, increase or potentiate the acetyl transferase activity of MCM3AP.

Following identification of a modulator, the substance may be purified and/or investigated further and/or manufactured. A modulator may be used to obtain peptidyl or non-peptidyl mimetics, e.g. by methods well known to those skilled in the art and discussed herein. It may be used in a therapeutic context as discussed below.

One class of putative modulator compounds can be derived from

the MCM3AP polypeptide sequence and/or a ligand which to which it binds, such as MCM3 or histone H3. Peptide fragments of these polypeptides or alleles, mutants or derivatives of such fragments are described herein. Nucleic acid encoding such peptides, vectors and host cells containing such nucleic acid, and methods of expressing nucleic acid encoding such peptides are further aspects of the present invention.

Other agents according to the present invention useful in modulating acetylation of MCM3 or histone H3 and therefore one or more of their functions modulate the acetyltransferase activity of the MCM3AP acetylase. Such agents may specifically inhibit the ability of MCM3AP to acetylate histone H3 or MCM3. Assays and screens for such agents are provided in accordance with the present invention, along with the agents themselves and their use in modulating the acetylation and function of MCM3 and/or histone H3.

An agent able to inhibit acetylation of MCM3 or histone H3 by MCM3AP may include another substance able to affect the catalytic properties of the enzymatically active site of the acetylase. An inhibitor of acetylation may interact with MCM3AP within the acetylase domain. Residues within this domain are involved with interaction with MCM3 and histone H3 and catalysis of the acetylation. Residues outside of the domain may also be involved in interacting with MCM3 and histone H3 and agents which interfere with such interaction may affect the acetylation as discussed elsewhere herein.

Agents useful in accordance with the present invention may be identified by screening techniques which involve determining whether an agent under test inhibits or disrupts the acetylation by an MCM3AP polypeptide (e.g. including the active acetylase domain,) of , with a histone H3 or MCM3

polypeptide.

MCM3AP polypeptides may be fragments of full length MCM3AP which retain the acetylase activity of the full-length polypeptide and are capable of acetylating substrate proteins. MCM3 and histone H3 polypeptide may be fragments which contain the residues which are acetylated by MCM3AP in the full-length polypeptide, and are themselves capable of being acetylated by MCM3AP. Smaller fragments, and analogues and variants of these fragment may similarly be employed, e.g. as identified using techniques such as deletion analysis or alanine scanning.

The present invention provides a peptide fragment of MCM3AP which is able to interact with histone H3 and/or inhibit interaction between MCM3AP and histone H3, and also a peptide fragment of MCM3AP which is able to inhibit acetylation of histone H3 and/or MCM3 by MCM3AP.

The present invention also provides a peptide fragment of histone H3 which is able to interact with MCM3AP and/or inhibit interaction between MCM3AP and histone H3 and also provides a peptide fragment of MCM3 or histone H3 which is able to inhibit acetylation of histone H3 and/or MCM3 by MCM3AP.

Such peptide fragments may be obtained by means of deletion analysis and/or alanine scanning of the relevant protein - making an appropriate mutation in sequence, bringing together a mutated fragment of one of the proteins with the other or a fragment thereof and determining interaction, preferably acetylation of MCM3 or histone H3 polypeptide. In preferred embodiments, the peptide is short, as discussed below, and may be a minimal portion that is able to interact with the

relevant counterpart protein and/or inhibit the relevant interaction. The invention further provides peptide fragments of MCM3 which are able to inhibit acetylation of MCM3 at the relevant residues and peptide fragments of histone H3 which are able to inhibit acetylation of histone H3 at the relevant residues.

Other proteins may bind histone H3 or MCM3 at an acetylation site, and may bind or not depending on whether the site is acetylated or not. A protein other than MCM3AP may bind at any one or more of the relevant lysines when they are acetylated, but not when not acetylated. A protein other than MCM3AP may bind at any one or more of the relevant lysines when they are not acetylated, but not when acetylated. Such proteins may be identified using standard methodology to identify interacting proteins. For instance, non-acetylated MCM3 or histone H3 fragments may be used in two-hybrid screens and chemically acetylated peptides may be screened against peptide and protein libraries.

The invention further extends to the use of MCM3 and peptide fragments thereof including one or more of the relevant lysines, acetylated or not acetylated, for obtaining a peptide or protein (other than MCM3AP) which binds at an acetylation site, particular a peptide or protein which binds or not depending on whether the site is acetylated or not. Further aspects of the invention provide assay methods for such peptides and proteins based on determining binding to MCM3 or a peptide fragment thereof, acetylated or not acetylated at one or more of the relevant lysines. The invention further extends to assays for substances able to modulate interaction of such peptides or proteins with the relevant acetylation site, and to methods of modulating such interaction, also modulating agents.

Peptide and polypeptide fragments of the present invention may include active portions, derivatives and functional mimetics of the MCM3AP, MCM3 or histone H3 peptides and polypeptides. An "active portion" of a polypeptide means a peptide which is less than said full length polypeptide, but which retains the biological activity of the full-length polypeptide i.e. the modulation of acetylation of histone H3 and/or MCM3 by MCM3AP.

A "fragment" of a polypeptide generally means a stretch of amino acid residues of at least about five contiguous amino acids, often at least about seven contiguous amino acids, typically at least about nine contiguous amino acids, more preferably at least about 13 contiguous amino acids, and, more preferably, at least about 20 to 30 or more contiguous amino acids. Fragments of the MCM3AP polypeptide sequence, the histone H3 polypeptide sequence and the MCM3 polypeptide sequence may include antigenic determinants or epitopes useful for raising antibodies to a portion of the amino acid sequence. Alanine scans are commonly used to find and refine peptide motifs within polypeptides, this involving the systematic replacement of each residue in turn with the amino acid alanine, followed by an assessment of biological activity.

Peptides in accordance with the present invention tend to be short, and may be about 40 amino acids in length or less, preferably about 35 amino acids in length or less, more preferably about 30 amino acids in length, or less, more preferably about 25 amino acids or less, more preferably about 20 amino acids or less, more preferably about 15 amino acids or less, more preferably about 10 amino acids or less, or 9, 8, 7, 6, 5 or less in length. Peptides according to the present invention may be about 10-40 amino acids in length,

about 5-10, about 10-15, about 10-20, about 10-30, about 20-30, or about 30-40 amino acids in length. Peptides which are MCM3 or histone H3 fragments may include one or more of the relevant lysine residues.

The present invention also encompasses peptides which are sequence variants or derivatives of a wild type MCM3AP, MCM3 or histone H3 sequence. Peptides which are variants of MCM3AP and histone H3 retain the ability to modulate interaction between MCM3AP and histone H3, particularly acetylation of histone H3 by MCM3AP, and/or ability to modulate acetylation of MCM3 by one or more other acetylases. Peptides which are variants of MCM3 retain the ability to modulate acetylation of MCM3 by MCM3AP, and/or ability to modulate acetylation of MCM3 by one or more other acetylases

Instead of using a wild-type MCM3AP, MCM3 or histone sequence, a peptide or polypeptide as described herein may include an amino acid sequence which differs by one or more amino acid residues from the wild-type amino acid sequence, by one or more of addition, insertion, deletion and substitution of one or more amino acids. Thus, variants, derivatives, alleles, mutants and homologues, e.g. from other organisms, are included.

Preferably, the amino acid sequence shares homology with the relevant MCM3AP, MCM3 or histone H3 sequence as referenced herein, preferably at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85%, 90% or 95% homology. Thus, a peptide fragment of MCM3AP, MCM3 or histone H3 may include 1, 2, 3, 4, 5, greater than 5, or greater than 10 amino acid alterations such as substitutions with respect to the wild-type sequence.

A derivative of a peptide for which the specific sequence is disclosed herein may be in certain embodiments the same length or shorter than the specific peptide. In other embodiments the peptide sequence or a variant thereof may be included in a larger peptide, as discussed above, which may or may not include an additional portion of MCM3AP, MCM3 or histone H3 sequence. 1, 2, 3, 4 or 5 or more additional amino acids, adjacent to the relevant specific peptide fragment in MCM3AP, MCM3 or histone H3, or heterologous thereto may be included at one end or both ends of the peptide.

Derivatives of peptides include the peptide linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule, and/or a targeting molecule such as an antibody or binding fragment thereof or other ligand. Techniques for coupling to both peptidyl and non-peptidyl coupling partners are well known in the art. In one embodiment, the carrier molecule is a 16 amino acid peptide sequence derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin@), which can be coupled to a peptide via a terminal Cys residue. The APenetratin@ molecule and its properties are described in WO 91/18981.

Various aspects of the present invention provide a substance, which may be a single molecule or a composition including two or more components, which includes a or polypeptide or peptide fragment of MCM3AP, particularly within the MCM3AP acetylase domain, or MCM3 or histone H3, particularly within the acetylated region of MCM3 or histone H3, a peptide consisting essentially of such a sequence, a peptide including a variant, derivative or analogue sequence, or a non-peptide analogue or mimetic which has the ability to interact with MCM3AP or MCM3 and/or modulate, disrupt or interfere with interaction between

MCM3AP and MCM3 or histone H3.

Variants include peptides in which individual amino acids can be substituted by other amino acids which are closely related as is understood in the art and indicated above. Non-peptide mimetics of peptides are discussed further below.

As noted, a polypeptide or peptide according to the present invention and for use in various aspects of the present invention may include or consist essentially of a fragment of MCM3AP, MCM3 or histone H3. Where one or more additional amino acids are included, such amino acids may be from MCM3AP, MCM3 or histone H3 or may be heterologous or foreign to MCM3AP, MCM3 or histone H3. A peptide may also be included within a larger fusion protein, particularly where the peptide is fused to a non-MCM3AP, non-MCM3 or non-histone H3 (i.e. heterologous or foreign) sequence, such as a polypeptide or protein domain.

A peptide fragment of MCM3, histone H3 or MCM3AP employed in accordance with the present invention may be a natural or synthetic chemical compound.

A modulator, agent or compound identified by any one of the methods provided by the present invention may be isolated and/or purified and/or further investigated and/or manufactured. Various methods and uses of such compounds are discussed elsewhere herein.

The skilled person can use the techniques described herein and others well known in the art to produce large amounts of peptides, for instance by expression from encoding nucleic acid.

Peptides can also be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

The present invention also provides nucleic acid sequences encoding peptides of the present invention.

As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Homology may be taken over the full-length of a sequence or over a part, such as 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, 200 contiguous nucleotides or amino acids. That two nucleotide sequences are said to share "homology" or be "homologous" is based on sequence comparison. Any

phylogenetic relationship is irrelevant for this. Those skilled in the art routinely refer to homology between nucleotide sequences with no implication for evolutionary origin. Two homologous nucleotide sequences may also be said to be "similar" or have a certain percentage similarity or a certain percentage identity.

In general, it is not critical which of the various standard algorithms are used to determine how homologous two nucleotide sequences are with one another. A preferred algorithm may be GAP, which uses the alignment method of Needleman and Wunsch (*J. Mol. Biol.* (1970) *48*, 443-453) and is included in the Program Manual or the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA). In the absence of instructions to the contrary, the skilled person would understand to use the default parameters with the aim of maximizing alignment, with a gap creation penalty = 12 and gap extension penalty = 4.

As described above, similarity or homology (the terms are used interchangeably) or identity may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, or BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are preferably set, using the default matrix, as follows: Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA; Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA; KTUP word length: 2 for proteins / 6 for DNA.

Nucleic acid sequence homology may be determined by means of

selective hybridisation between molecules under stringent conditions.

Preliminary experiments may be performed by hybridising under low stringency conditions. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For example, hybridisations may be performed, according to the method of Sambrook et al. (below) using a hybridisation solution comprising: 5X SSC (wherein >SSC= = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt=s reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridisation is carried out at 37-42°C for at least six hours. Following hybridisation, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): T_m = 81.5°C + 16.6 Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex.

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57□C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence

identity would be observed using a hybridisation temperature of 42□C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridisation overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridisation overnight at 65oC in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the (e.g. human) genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA.

Nucleic acid sequences encoding the peptides or polypeptides of the present invention may be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, A Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992). These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such

nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding the peptides may be generated and used in any suitable way known to those of skill in the art, including taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers.

Modifications to a nucleic acid sequence may be made, e.g. using site directed mutagenesis, to lead to the production of modified peptide, e.g. an allele or mutant form of a peptide, or to take account of codon preference in the host cells used to express the nucleic acid.

In order to obtain expression of the nucleic acid sequences of the invention, the sequences may be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to control its expression. Vectors may be chosen or constructed, They may contain appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate, e.g. nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the peptide produced in the host cell is secreted from the cell. Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for

example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel *et al.* eds., John Wiley & Sons, 1992.

Peptide may then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the peptide is produced and recovering the peptide from the host cells or the surrounding medium.

A further aspect provides a method which includes introducing a nucleic acid molecule of the invention into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as 'transformation', may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the

encoded peptide is produced. If the peptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a peptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction into target cells of nucleic acid encoding a peptide of the present invention may take place *in vivo* by way of gene therapy, for instance to modulate e.g. disrupt or interfere with, binding of MCM3AP to a MCM3 or histone H3 and thereby to disrupt acetylation.

Systems for cloning and expression of peptides in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous peptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*.

In light of the above, the present invention also provides a method of making a polypeptide or peptide as described herein, in particular a MCM3AP polypeptide, the method including causing or allowing expression from nucleic acid encoding the peptide.

This may conveniently be achieved by growing a host cell containing the nucleic acid in culture under appropriate

conditions which cause or allow expression of the peptide. Note however that expression may also be carried out in *in vitro* systems, e.g. reticulocyte lysate.

Following production of an MCM3AP polypeptide, it may be tested for acetyltransferase activity, e.g. by determination of acetylation of a lysine residue of histone H3 or MCM3 polypeptide on incubation of the polypeptide with the histone H3 or MCM3 polypeptide.

A further aspect of the present invention provides a host cell containing heterologous nucleic acid as disclosed herein.

The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

Thus, a host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell (which introduction may take place in vivo or ex vivo), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or a bird, such as a chicken.

Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention.

Animal models may also be useful for any of the various diseases discussed elsewhere herein.

This may have a therapeutic aim. (Gene therapy is discussed elsewhere herein). Also, the presence of a mutant, allele, derivative or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying substances which modulate activity of the encoded peptide *in vitro* or are otherwise indicated to be of therapeutic potential. Knock-out mice, for instance, may be used to test for radiosensitivity. Conveniently, however, at least preliminary assays for such substances may be carried out *in vitro*, that is within host cells or in cell-free systems. Where an effect of a test compound is established on cells *in vitro*, those cells or cells of the same or similar type may be grafted into an appropriate host animal for *in vivo* testing.

For instance, MCM3AP function or activity may be measured in an animal system such as a tumour model, e.g. involving a xenograft, relying on active MCM3AP.

An assay or screening method according to the present invention may thus take the form of an *in vivo* assay.

Methods of determining the interaction of MCM3AP of histone H3 or the acetylation of MCM3 and histone H3 by MCM3AP and of screening for an agent able to modulate the interaction of an MCM3AP to a MCM3 or histone H3, include methods in which a suitable end-point is used to assess interaction.

Suitable end points include the determination of the

acetylation of an MCM3 or histone H3 polypeptide by an MCM3AP polypeptide using methods described herein.

Alternatively, interaction may be determined by any number of techniques known in the art, qualitative or quantitative. They include techniques such as radioimmunoassay, co-immunoprecipitation, scintillation proximity assay and ELISA methods.

Binding of MCM3AP to a binding partner such as MCM3 or histone H3 may be studied by labelling either one with a detectable label and bringing it into contact with the other which may have been immobilised on a solid support.

Suitable detectable labels, especially for peptidyl substances include ^{35}S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as fusion proteins containing an epitope which can be labelled with an antibody. Acetylated polypeptides may be detected using a ^{14}C acetyl label.

The polypeptide or peptide which is immobilized on a solid support may be immobilized using an antibody against that polypeptide bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion peptide including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test modulator can be assayed by determining its ability to diminish the amount of labelled peptide (e.g. labelled MCM3 or histone H3) which binds to the immobilized GST-fusion peptide (e.g. immobilised fusion peptide of GST and a peptide comprising an MCM3AP). This may be determined by fractionating

the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound peptide and the amount of peptide which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

Binding or interaction of an MCM3AP polypeptide with a binding partner such as an MCM3 or histone H3 polypeptide may also be determined using a two-hybrid assay.

For example, an MCM3AP or binding partner polypeptide may be fused to a DNA binding domain such as that of the yeast transcription factor GAL4. The GAL4 transcription factor includes two functional domains. These domains are the DNA binding domain (GAL4DBD) and the GAL4 transcriptional activation domain (GAL4TAD). By fusing an MCM3AP polypeptide to one of those domains, and binding partner such as MCM3 polypeptide to the respective counterpart, a functional GAL4 transcription factor is restored only when the two peptides interact. Thus, interaction of these peptides may be measured by the use of a reporter gene linked to a GAL4 DNA binding site which is capable of activating transcription of said reporter gene.

This two hybrid assay format is described by Fields and Song, 1989, Nature 340; 245-246. It can be used in both mammalian cells and in yeast. Other combinations of DNA binding domain and transcriptional activation domain are available in the art and may be preferred, such as the LexA DNA binding domain and the VP60 transcriptional activation domain.

When looking for substances which interfere with the interaction of MCM3AP with a binding partner such as MCM3 or Histone H3, and which may therefore modulate the acetylation

of MCM3 and/or histone H3 by MCM3AP, a peptide fragment of MCM3AP or binding partner may be employed as a fusion with (e.g.) the LexA DNA binding domain, and the counterpart peptide fragment containing MCM3AP or binding partner as a fusion with (e.g.) VP60. An expression cassette may be used to express a test peptide within a host cell.

The expression cassette may be one vector from a library of expression vectors which encode a diverse range of peptides. A reduction in reporter gene expression (e.g. in the case of β-galactosidase a weakening of the blue colour) results from the expression of a peptide which disrupts the MCM3AP/binding partner interaction, which interaction is required for transcriptional activation of the β-galactosidase gene. Where a test substance is not peptidyl and may not be expressed from encoding nucleic acid within a said third expression cassette, a similar system may be employed with the test substance supplied exogenously.

The end-point of an in vivo assay, that is to say the property which is determined in order to assess the interaction of MCM3AP with a binding partner such as MCM3 or histone H3 (e.g. to assess whether a test substance interacts with MCM3AP or whether a test agent may affect the binding of MCM3AP to MCM3 or histone H3) may be the acetylation of the MCM3 and/or the histone H3. Methods for assaying the acetylation of a protein as well known to those skilled in the art. As noted, those skilled in the art well appreciate the need for and design of appropriate controls for validation of results.

In accordance with the present disclosure, MCM3AP may be used as a binding partner in any method of determining acetylation.

As other end points for *in vivo* assays employing MCM3AP, the

effect on RNA transcription, G_1/S phase checkpoint arrest, DNA replication inhibition, cell viability, cell killing and so on, may be measured. Suitable methods are known to those skilled in the art.

Acetylation may be determined by any convenient method known to a skilled person. For example, MCM3 polypeptide or histone H3 polypeptide may be immobilised e.g. on a bead or plate, and acetylation detected using an antibody or other binding molecule which binds the relevant site of acetylation with a different affinity when the site is acetylated from when the site is not acetylated. Such antibodies may be obtained by means of any standard technique as discussed elsewhere herein, e.g. using a acetylated peptide (such as a fragment of MCM3). Binding of a binding molecule which discriminates between the acetylated and non-acetylated form of MCM3 polypeptide or histone H3 polypeptide may be assessed using any technique available to those skilled in the art, which may involve determination of the presence of a suitable label.

Acetylation may also be assayed in solution, e.g. as described in Bannister and Kouzarides (1996), *Nature*, **384**: 641-643. Briefly, protein substrate (~1µg) and ~0.1 pmol of acetyltransferase are mixed to give a final volume of 30µl in buffer IPH (50mM Tris.HCl pH8.0, 150mM NaCl, 5mM EDTA, 0.5% [v/v] NP-40, 0.1mM PMSF). Reactions are initiated by the addition of [14-C] - acetyl CoA (1.85 kBq: 1.85 GBq/mmol; Amersham) and incubated at 30□C for 10-45 min. The reaction products are then resolved by SDS-PAGE and viewed following fluorography of the gel. Alternatively, following SDS-PAGE, the resolved proteins can be Western blotted to a nitrocellulose membrane, which is then dried and exposed to film.

A further option is an in-gel activity assay, such as described by Brownell and Allis (1995), *Proc. Natl. Acad. Sci.*, **92**: 6364-6368 or Mizzen, *et al* (1996), *Cell*, **87**: 1261-1270. Samples may be crude cellular extracts, partially purified fractions, highly purified cellular proteins or bacterially produced and purified recombinant proteins. Before loading onto the activity gel the sample is made to 1X SDS-PAG loading buffer and boiled for 2 minutes. The gel is a standard Laemmli SDS-PAG except that purified protein substrate is added to the resolving gel to a final concentration of 1 mg/ml. Polymerisation of the gel is initiated using standard techniques, at which point the protein substrate becomes immobilized within the gel matrix. After adding the stacking gel, the samples are loaded and the gel run as a standard SDS-PAG. After the gel has run it is soaked, with gentle agitation, in 100 ml of wash buffer (50mM Tris.HCl pH 8.0, 0.1% β-mercaptoethanol) containing 20% (v/v) isopropanol for 20 minutes at room temperature. This washing step is repeated twice. Proteins in the gel are then denatured by washing in 100 ml of wash buffer containing 8M urea for 20 minutes at room temperature. This denaturing step is repeated twice. The gel is then soaked without agitation in 100 ml of wash buffer containing 0.04% tween-40 for 20 minutes at 4°C. This step is then repeated but for a duration of 12 hours, after which the gel is washed twice for a period of 20 minutes each time. After the final soak the gel/buffer is allowed to slowly come to room temperature. The gel is washed in wash buffer containing 10% (v/v) glycerol for 20 minutes at room temperature. The gel is then placed in a heat sealable bag and 3 ml of the same buffer containing 10μCi of [3H]-acetylCoA is added. The contents are thoroughly mixed, air bubbles removed and the bag sealed. The reaction is then performed by immersing the bag in a 30°C water-bath for at least 30 minutes. Following the acetylation step, the gel is

recovered and washed extensively in several 100 ml changes of gel destain solution (10% [v/v] methanol, 10% [v/v] acetic acid). This washing stage is performed at room temperature with agitation and should include an overnight wash.

Of course, the person skilled in the art will design any appropriate control experiments with which to compare results obtained in test assays.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive for ability to modulate interaction between MCM3AP and histone H, modulate acetylation of histone H3 and/or MCM3 by MCM3AP and/or modulate acetylase activity of MCM3AP or a mediated activity. Following identification of a suitable agent, it may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Antibodies directed to the site of binding in MCM3AP, MCM3 or histone H3 form another class of putative modulators of MCM3AP acetylase activity. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the binding.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with MCM3 or histone H3 or a peptide fragment thereof or MCM3AP or a fragment thereof. Antibodies may be

obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage *et al.*, 1992, Nature **357**: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for either MCM3AP, MCM3 or histone H3 may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and

VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may

directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule. The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies may also be used in purifying and/or isolating a MCM3AP polypeptide, or a binding partner such as MCM3 or histone H3 polypeptide, for instance following production of the peptide by expression from encoding nucleic acid. Antibodies may be useful in a therapeutic context (which may include prophylaxis) to disrupt binding of an MCM3AP to MCM3 or histone H3 with a view to inhibiting the acetylation activity of MCM3AP. Antibodies can for instance be micro-injected into cells, e.g. at a tumour site, subject to radio- and/or chemo-therapy (as discussed already above). Antibodies may be employed in accordance with the present invention for other therapeutic and non-therapeutic purposes which are discussed elsewhere herein.

As noted, the agent may be peptidyl, e.g. a peptide which includes a sequence as recited above, or may be a functional analogue of such a peptide.

As used herein, the expression "functional analogue" relates to peptide variants or organic compounds having the same functional activity as the peptide in question, which may interfere with the acetylation of MCM3 or histone H3 by MCM3AP. Examples of such analogues include chemical compounds which are modelled to resemble the three dimensional structure

of the MCM3AP or MCM3 domain in the contact area, and in particular the arrangement of the key amino acid residues as they appear in MCM3AP or MCM3.

In a further aspect, the present invention provides the use of a peptide fragment of MCM3 or histone H3 which is capable of binding to MCM3AP , in a method of designing a peptide or non-peptidyl mimetic of MCM3 or histone H3, which mimetic is able to bind to MCM3AP and modulate the acetylation of MCM3 or histone H3 by MCM3AP.

The present invention similarly provides for the use of MCM3AP or fragment thereof in a method of designing a peptide or non-peptidyl mimetic of MCM3AP, which mimetic is able to bind to MCM3 or histone H3 and modulate the acetylation of MCM3 or histone H3 by MCM3AP.

Accordingly, the present invention provides a method of designing a mimetic of MCM3 or histone H3 which has the biological activity of binding to MCM3AP, or a method of designing a mimetic of MCM3AP which has biological activity of binding to MCM3 or Histone H3, said method comprising:

(i) analysing a substance having the biological activity to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

Suitable modelling techniques are known in the art. This includes the study of the bonding between MCM3AP and MCM3 or histone H3 and to design compounds which contain functional groups arranged in such a manner that they could reproduced that bonding.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, for instance MCM3 or Histone H3 may not be well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of the above approach, the three-dimensional structure of a ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A compound found to have the ability to affect MCM3AP activity has therapeutic and other potential in a number of contexts, as discussed. For therapeutic treatment such a compound may be used in combination with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy. In such a case, the assay of the invention, when conducted *in vivo*, need not measure the degree of modulation of acetylation of MCM3 and/or histone H3 (or appropriate fragment, variant or derivative thereof) by MCM3AP or the modulation of acetylase activity of MCM3AP caused by the compound being tested.

Instead, the effect on transition of cells into S-phase may be determined. It may be that such a modified assay is run in parallel with or subsequent to the main assay of the invention in order to confirm that any such effect is as a result of the inhibition of interaction between MCM3AP and MCM3 or histone H3 caused by said inhibitor compound and not merely a general toxic effect.

Thus, an agent identified using one or more primary screens (e.g. in a cell-free system) as having ability to modulate the acetylase activity of MCM3AP and/or the acetylation of MCM3 and/or histone H3 may be assessed further using one or more secondary screens. A secondary screen may involve testing for a biological function of MCM3AP as noted above (e.g. induction of S-phase or repression of DNA replication).

The present invention further provides the use of a polypeptide or peptide which includes a sequence as disclosed, or a derivative, active portion, analogue, variant or mimetic, thereof able to interact with histone H3 and/or modulate, inhibit or potentiate, acetylation of histone H3 and/or MCM3

by MCM3AP and/or modulate, inhibit or potentiate, MCM3AP acetyltransferase activity, in screening for a substance or compound able to interact with histone H3 and/or modulate, inhibit or potentiate, acetylation of histone H3 and/or MCM3 by MCM3AP and/or modulate, inhibit or potentiate, MCM3AP acetyltransferase activity.

Generally, such a substance according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients. As noted below, a composition according to the present invention may include in addition to an modulator compound as disclosed, one or more other molecules of therapeutic use, such as an anti-tumour agent.

The invention further provides a method of treatment which includes administering to a patient a composition comprising an agent which interferes with the acetylation of MCM3 by MCM3AP, for example for the treatment of a disorder related to cellular proliferation. Exemplary purposes of such treatment are discussed elsewhere herein.

The invention further provides various therapeutic methods and uses of one or more substances selected from (i) a peptide fragment of MCM3 or histone H3 polypeptide which is able to bind to MCM3AP; (ii) a MCM3AP polypeptide (e.g. one described herein or identified by a screening method of the present invention); (iii) a modulator identified by a screening method of the present invention; (iv) a mimetic of any of the above substances which can bind to an MCM3AP or MCM3 or histone H3.

The therapeutic/prophylactic purpose of such a method or use may be the modulation, e.g. disruption or interference, of the interaction of MCM3AP with MCM3 or histone H3, e.g. to modulate the acetylation which is mediated by virtue of such interaction.

The therapeutic/prophylactic purpose may be:

(i) Cancer treatment, which may for example be in combination with chemotherapy and/or radiotherapy.

(ii) Cancer prophylaxis,

(iii) Treatment of other proliferative disorders e.g. psoriasis, cataracts;

In various further aspects, the present invention thus provides a pharmaceutical composition, medicament, drug or other composition for such a purpose, the composition comprising one or more such substances, the use of such a substance in a method of medical treatment, a method comprising administration of such a substance to a patient, e.g. for treatment(which may include preventative treatment) of a medical condition, e.g. a condition associated with a defect or disorder in transcriptional control, DNA replication, or cell cycle control, e.g. for treatment of a disorder of cellular proliferation such as cancer, use of such a substance in the manufacture of a composition, medicament or drug for administration for such a purpose, e.g. for treatment of a proliferative disorder, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

The substances may be used as sole active agents or in combination with one another or with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy.

Whatever the substance used in a method of medical treatment of the present invention, administration is preferably in a 'prophylactically effective amount' or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A substance or composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated, e.g. cancer.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Liposomes, particularly cationic liposomes, may be used in carrier formulations.

Examples of techniques and protocols mentioned above can be found in Remington=s Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The substance or composition may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells.

Targeting therapies may be used to deliver the active

substance more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering such substances directly, they may be produced in the target cells by expression from an encoding nucleic acid introduced into the cells, e.g. from a viral vector. The vector may be targeted to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

Nucleic acid encoding the substance e.g. a peptide able to modulate, e.g. interfere with, the interaction of MCM3AP with MCM3 or histone H3 and thereby affect the acetylation of MCM3 or histone H3 by MCM3AP, may thus be used in methods of gene therapy, for instance in treatment of individuals, e.g. with the aim of preventing or curing (wholly or partially) a disorder.

Vectors such as viral vectors have been used in the prior art to introduce nucleic acid into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired peptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors,

are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

As an alternative to the use of viral vectors in gene therapy other known methods of introducing nucleic acid into cells includes mechanical techniques such as microinjection, transfer mediated by liposomes and receptor-mediated DNA transfer.

Receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells, is an example of a technique for specifically targeting nucleic acid to particular cells.

The discovery of the acetylase activity of MCM3AP and its role in DNA replication and cellular proliferation paves the way for aspects of the present invention to provide the use of materials and methods, such as are disclosed and discussed above, for establishing the presence or absence in a test sample, for example obtained from an individual, of aberrant, i.e. increased, reduced or abolished MCM3AP acetylase activity. Such aberrant activity may be for diagnosing a predisposition of an individual to a condition associated with cellular proliferation or for diagnosing an individual as suffering from a condition associated with cellular proliferation, such as cancer.

Aberrant expression may be detected at the protein level, by determining the acetylase activity of MCM3AP, as described

herein, for example, the presence or absence or amount of acetylase activity or at the nucleic acid level (i.e. DNA or RNA), by determining the presence of a mutant, variant or allele of the MCM3AP gene which encodes a MCM3AP protein which has aberrant activity or which expresses aberrant i.e. abolished, reduced or increased levels of MCM3AP protein. The presence or amount of MCM3AP expression may be determined by determining the presence and/or amount of MCM3AP mRNA.

A peptide or other substance having an ability to modulate or interfere with the interaction of MCM3AP with MCM3 or histone H3, a nucleic acid molecule which encodes a peptide having that ability, may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

In still further aspects the present invention provides for the purification of MCM3AP or a fragment thereof, or for the purification of histone H3 or a fragment of either of these which has the ability to bind to MCM3AP. The invention also provides for a purified MCM3AP protein. The purified polypeptide may be about 10% pure, more preferably about 20% pure, more preferably about 30% pure, more preferably about 40% pure, more preferably about 50% pure, more preferably about 60% pure, more preferably about 70% pure, more preferably about 80% pure, more preferably about 90% pure, more preferably about 95% pure, or substantially pure.

In another aspect the present invention provides a method of purifying MCM3AP, the method including contacting the MCM3AP with histone H3 or a fragment thereof.

A mixture of material including MCM3AP may be contacted

against immobilised phosphopeptide (e.g. immobilised either covalently or non-covalently such as via a specific binding molecule such as streptavidin or biotin) and molecules which do not bind to the phosphopeptide are washed off.

Likewise, the invention provides a method of purifying histone H3, the method including contacting material containing the phosphopeptide with MCM3AP or a fragment thereof which binds histone H3.

Following purification, the MCM3AP or histone H3 may be used as desired, e.g. in an assay for an agent which modulates its activity, e.g. binding, in raising or obtaining a specific antibody or other binding molecule, or in a therapeutic context.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure, in which all documents cited are incorporated by reference.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figure described below.

Figure 1 shows a model for the cycle of MCM3 modification, which is regulated by cell cycle. Although the present invention is not in any way bound by theory, the model shows the binding of MCM3AP to MCM3 in the cytoplasm and the importation of MCM3AP into the nucleus in an MCM3-dependent manner.

Figure 2 shows the results of *in vitro* DNA replication assays using mimosine treated HeLa cell nuclei and cytosol prepared

from wild-type MCM3AP and acetylase defective MCM3AP mutant transfected 293T cells.

Figure 3 shows the results of *in vivo* DNA replication assays in 293T cells. The proportion of GFP positive cells that were also BrdU positive is shown.

Experimental

Cells, Plasmids, and Recombinant Proteins.

HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Sf9 insect cells were cultured in Grace's insect medium with 10% fetal calf serum or in SF-900 II medium (GIBCO-BRL).

The full length cDNA of human MCM3 was inserted into the pcDNA3.1 vector (STRATAGENE). The cDNA of SV40 large T antigen-encoding NLS was prepared by PCR of pGAD424 (Clontech) [Takei, 1998]. The amplified cDNA containing the NLS and a Gal4 activation domain was inserted into the pcDNA3.1 vector. The full length cDNA of human MCM3AP was inserted into pEGFP C2 vector (Clontech). These plasmids were transfected into HeLa cells using Effectene Transfection Reagent (QIAGEN). The transfected cells were incubated for 20 hours and assayed by microscopy. For indirect immunofluorescence microscopy, the exponentially growing HeLa cells or the transfected HeLa cells were fixed with methanol and acetone (1:1) mixture for 2 min. The fixed cells were washed with PBS and incubated with anti-MCM3AP antibody or anti-His_6 antibody (Sigma) for 1 hour at room temperature. These cells were further treated with Cy3-labeled secondary antibody for 30 min and then analysed by confocal microscopy.

For expression of human MCM2, MCM3, and MCM3AP, the full length cDNAs of these proteins were inserted into pAcHLT

vector (PHARMINGEN), respectively. These plasmids were co-transfected with BaculoGold DNA (PHARMINGEN) into Sf9 insect cell line with Superfectin transfection reagent (GIBCO-BRL) to generate recombinant baculoviruses. The recombinant viruses were co-cultured with Sf9 at 27°C. After 48 hours these cells were harvested and crushed by sonication in sonication buffer (10 mM Hepes-Na, pH 7.4, 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 5 mM imidasol, and protease inhibitors). The cell extracts were obtained as a centrifugation supernatant at 10000 x g for 20 min. The extracts were applied on the TALON Superflow metal affinity column (Clontech) equilibrated with the sonication buffer. The columns were washed extensively with wash buffer (20 mM Hepes-Na, pH7.4, 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 10 mM imidasol, 10% glycerol and protease inhibitors) including 500 mM NaCl or 0.1% Triton X100, sequentially. The fractions eluted with wash buffer including 50 mM imidasol were diluted 10 times with the sonication buffer and applied on the new TALON Superflow metal affinity column. The columns were washed and eluted as described above.

To make mutant MCM3AP inserted into pGAD424 (Clontech), mutant MCM3AP cDNA was cut out from pAcHLT then inserted into pGAD424 at *BamH1* and *Sal1* sites. The yeast strain Y190 (Clontech) has the genotype his3-200, lys2-801, LYS2::$GAL1_{UAS}$-HIS_{TATA}-HIS3. The method for transfection of plasmids to Y190 is described in Takei Y. and Tsujimoto G. (1998).

Cells were labelled with 50μM BrdU for 1.5 h, 20 h after transfection, then fixed with 2% paraformaldehyde and denatured in 50 mM NaOH for 5 min. Incorporated BrdU was probed with anti-BrdU antibody (Amersham Pharmacia) and visualised by Texas Red labelled secondary antibody. Chromatin was stained by TOTO3 dye. Cells were observed with confocal

microscopy and TOTO3 positive images were recognised as cells. The GFP positive and BrdU positive cells were counted to estimate the proportion of DNA-replicating cells. At least 100 GFP positive cells in each sample were observed and the experiment was repeated three times.

Acetyltransferase assay

For acetylation of histones and purified individual histone the 1 µg of histones (Sigma) or 0.5 µg of monomer histone (Roche) was incubated with 0.5 µg purified MCM3AP and 40 µM acetyl CoA in an acetylation buffer consisting of 50 mM Tris HCl, pH 8.0, 0.1 mM EDTA, 10 mM Na-butyrate, 1 mM DTT, 10% glycerol.

For acetylation of MCM3, 0.5 µg of purified recombinant MCM3 was incubated with 0.1 µg of purified MCM3AP and 20 µM acetyl CoA in the acetylation buffer. After the incubation at 30°C for 1 hour the samples were separated by SDS-PAGE and transferred to a PVDF membrane at a constant voltage of 15 V for 1 h. The membrane was blocked with 3% BSA and 0.25% Triton X 100 in PBS and probed with anti-acetylated Lys antibody (NEB) in 3% BSA and 0.25% Triton X 100 in PBS. The bound antibodies were detected with peroxidase-conjugated second antibody (Amersham Pharmacia) using 1/5000 dilution in 3% BSA and 0.25% Triton X 100 in PBS and the protein bands were visualized by the ECL PLUS kit (Amersham Pharmacia).

For acetylation with the ^{14}C acetyl CoA, 5µg of recombinant MCM3 was incubated with MCM3AP (0.5µg) in the presence of ^{14}C acetyl CoA (Amersham Pharmacia) in the acetylation buffer. After 1 hr incubation at 30°C, samples were analysed by SDS-PAGE and blotted to PVDF membrane. The membrane was exposed to X-ray film for 5-7 days at -80°C.

For dot blots, after acetylation the reaction mixture was directly spotted onto nitocellulose membrane. The membrane was dried and incubated with 3% BSA in PBS, then probed with anti-acetyl Lys antibody (Upstate biotechnology) in 3% BSA in PBS. The bound antibodies were detected with peroxidase conjugated secondary antibody (Amersham Pharmacia)using 1/5000 dilution in 3% BSA in PBS and visualised by the ECL PLUS kit (Amersham Pharmacia).

Yeast Culture and Transfection.

The full length cDNA of the MCM3AP was inserted into pGBT9 (Clontech) to express a fusion protein of Gal4 DNA binding domain and MCM3AP. The plasmid was transfected into SFY526 (MATa, ura3-52, his 3-200, ade 2-101, lys 2-801, leu2-3, gal4-542, URA3::GAL1-lacZ) as suggested by the manufacturer. The transfected cells were cultured on minimal SD agar plate containing essential amino acids except for Leu.

Drug Treatment and Cell Extraction.

The exponentially growing HeLa cells were cultured with 0.5 mM mimosine or 50 ng/ml nocodazole in the growing medium. After incubation for 25 hours the cells were washed with ice cold PBS and used for preparation of cell extracts. Exponentially growing HeLa cells or drug-treated cells were harvested and washed with ice-cold hypotonic buffer (10 mM Hepes-KOH, pH7.3, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and protease inhibitors) [Coverley, 1998]. The cells were resuspended in the hypotonic buffer containing 1% NP40 and incubated on ice for 15 min. After incubation the cells were centrifuged at 15000 rpm for 5 min. The supernatant was stored at -80°C as the soluble protein fraction. The precipitate was washed with hypotonic buffer containing 1% NP40 and resuspended in hypotonic buffer containing 1% NP40 and 0.5 M NaCl. After incubation for 15 min on ice, the cells were centrifuged at 15000 rpm for 5 min.

The supernatant was stored at -80°C as the structure bound protein fraction.

For immunoprecipitation of MCM3, HeLa cells were broken by sonication in hypotonic buffer containing 1% NP40 and 0.5M NaCl. After incubation for 15 minutes on ice, the cells were centrifuged at 15 000 rpm for 5 minutes. The supernatant was diluted by hypotonic buffer 5 times to decrease the concentration of salt and detergent and then incubated with antibodies and protein A.

Results

A fusion protein of MCM3AP and GFP (GFP-MCM3AP) was over-expressed in HeLa cells. MCM3AP was expected to be localized in the cytoplasm, since we could not find a putative nuclear localization signal (NLS) in its primary sequence. The effect of MCM3 over-expression on the localization of MCM3AP was investigated, since MCM3 is consistently nuclear and it binds to the MCM3AP.

GFP-MCM3AP and His_6 tagged MCM3 were expressed respectively or co-expressed in HeLa cells. The GFP-MCM3AP was expressed in HeLa cells with the His_6 tagged SV40 large T antigen NLS as a control. These cells were fixed and incubated with anti His_6-tag antibody. The bound antibodies were detected by Cy3-labelled secondary antibody. Then the cells were analysed by confocal microscopy.

GFP-MCM3AP was observed to be clearly localized in the cytoplasm. The His_6 tagged MCM3 was localized in the nucleus. Co-expression of MCM3 changed the localization of the GFP-MCM3AP to the nucleus from the cytoplasm. However, the expression of the GFP-MCM3AP did not affect the localization of His_6 tagged MCM3.

Co-expression of SV40 large T antigen NLS had no effect on the localization of GFP-MCM3AP, while SV40 large T antigen NLS was localized in the nucleus as well as MCM3. These results indicate that the nuclear localization of MCM3AP is dependent on the nuclear import of MCM3.

Exponentially growing HeLa cells were also fixed and incubated with anti MCM3AP antibody. The bound antibodies were detected by Cy3-labelled secondary antibody. Then the cells were analysed by confocal microscopy. As a control, the 0.1 µg/ml of antigen peptide was added during the incubation with first antibody.

Indirect immunofluorescence microscopy with anti-MCM3AP antibody was used to examine whether endogenous MCM3AP is nuclear *in vivo* [Takei, 1998]. MCM3AP was observed in the nucleus as diffuse dots. In early G1 phase, the MCM3AP was already localized near or on the chromatin. The antibody signal disappeared on addition of excess antigen peptide. These results indicate that MCM3AP is a nuclear protein.

Recombinant MCM3AP protein was prepared and assayed for acetyltransferase activity with histones as a substrate.

Insect cells were infected with virus containing MCM3AP cDNA and allowed to express virus-encoded protein. The expressed MCM3AP was purified from cell extracts and 1 µg of purified MCM3AP was analysed by SDS PAGE (10%). The proteins were visualized by CBB staining.

The purified MCM3AP was incubated with both histones and acetyl CoA or one of these at 30°C. Histones were also incubated with acetyl CoA without the MCM3AP. After

incubation for 1 hour, the samples were analysed by SDS PAGE (15%) and blotted to PVDF membrane. The blotted membrane was incubated with anti-acetylated Lys antibody and the bound antibodies were detected by horse radish peroxidase-labelled secondary antibody and visualized with ECL PLUS kit.

The incubation of MCM3AP with histones and acetyl CoA revealed that MCM3AP possesses intrinsic acetyltransferase activity. The incubation of MCM3AP with either histones or acetyl CoA, or the incubation of histones and acetyl CoA without the MCM3AP failed to generate positive signals indicating that the signal observed was generated by MCM3AP-mediated histone acetylation.

The acetyltransferase assays were then repeated using purified individual histones. The individual histones, histone H2B, H3, and H4, were incubated respectively with or without MCM3AP at 30°C. After incubation for 1 hour the samples were analysed by SDS PAGE (15%).

MCM3AP was shown to be capable of acetylating only histone H3. MCM3AP addition greatly stimulated histone H3 acetylation compared to the faint background signal generated without MCM3AP. No MCM3AP-dependent acetylation of histone H4 or histones H2B or H2A was observed.

Since histone acetylation is implicated in the initiation of RNA transcription, the possibility that MCM3AP could activate the initiation of transcription was then assessed.

MCM3AP was expressed as a fusion protein with Gal4 DNA-binding domain in yeast which contains the Gal4-responsive element-β-galactosidase cDNA as a reporter gene by transforming the yeast SFY526 strain with pGBT9 or the vector containing fusion

construct. After incubation, the β-galactosidase activity of these colonies was analysed employing a colony-lift filter assay using 5-bromo-4-chloro-3 indolyl-β-D-galactosidase as substrate.

We observed β-galactosidase activity in the strain containing the MCM3AP : Gal4 DNA-binding domain fusion. We could not detect β-galactosidase activity in the strain which was transformed with mock vector.

MCM3AP was therefore shown to activate transcription of RNAs when the protein binds to the proper region of chromatin.

The effects of MCM proteins on histone acetylation by MCM3AP were assessed. Insect cells were infected with virus containing cDNA of MCM2 and MCM3, respectively, and allowed to express virus-encoded proteins. The expressed MCM2 and MCM3 were purified from cell extracts and 1 μg of purified MCM2 and MCM3 were analysed by SDS PAGE (10%). The proteins were visualized by CBB staining.

Histones were incubated with MCM3AP and acetyl CoA in the absence or presence of 1 μg of MCM2 or MCM3. After 1 hour, these samples were analysed by SDS PAGE (15%) and blotted to PVDF membrane. The acetylation of histones was then observed.

We did not observe any effects of MCM2 on histone acetylation by MCM3AP, although MCM2 has been reported to bind to histones with high affinity [Ishimi, 1996]. However MCM3AP-mediated histone acetylation was partially inhibited by MCM3.

The purified MCM3AP was incubated with both MCM3 and acetyl CoA or one of these at 30°C. MCM3 was also incubated with acetyl CoA without the MCM3AP. After incubation for 1 hour

the samples were analysed by SDS PAGE (7.5%).

MCM3 was detected by anti-acetylated Lys antibody only when MCM3 was incubated with both MCM3AP and acetyl CoA. The acetylation of MCM3 is completely dependent upon the presence of both acetyl CoA and MCM3AP, thus excluding the possibility that the acetylated MCM3 resulted from either a nonspecific interaction between MCM3 and acetyl CoA or MCM3 auto-acetylation.

Cellular protein fractions were prepared from soluble proteins (including dissolved membranes) and structure-bound proteins. MCM3 is known to bind to chromatin in G1 phase of the cell cycle and its binding is resistant to low concentrations of detergent but not to high concentrations of salt buffer. Thus, free MCM3 is fractionated into the soluble protein fraction and the chromatin-bound MCM3 is fractionated into the structure-bound protein fraction [Coverley, 1998].

The soluble protein fraction and the structure bound fraction prepared from 5 x 10^5 cells treated with nocodazole or mimosine or without any drugs were analysed by 7.5% SDS-PAGE. The proteins were blotted to PVDF membrane. The membranes were incubated with anti-MCM3 antibody and anti-acetylated Lys antibody, respectively. The bound antibody was detected by horse radish peroxidase-labelled secondary antibody and visualized by ECL PLUS kit.

An acetylated protein which exhibited the same mobility as MCM3 on SDS-PAGE was observed in the structure-bound protein fraction of asynchronized cells. This acetylated protein is detected in the structure-bound protein fraction of mimosine treated cells (late G1 phase) but not that of nocodazole treated cells (M phase). This behaviour of the acetylated

protein is similar to that of chromatin-bound MCM3.

The soluble protein fraction and structure bound fraction prepared from 1 x 10^5 cells treated with nocodazole or mimosine or without any drugs were analysed by 7.5% SDS-PAGE. The proteins were blotted to PVDF membrane. The membranes were incubated with the indicated antibodies, respectively. The bound antibody was detected by horse radish peroxidase-labelled secondary antibody and visualized by an ECL PLUS kit.

The localization of MCM3 was shown to be changed in a cell cycle dependent manner. The nocodazole treated cells were arrested in M phase and chromatin-bound MCM3 disappeared. On the other hand, mimosine treated cells were arrested in late G1 phase and chromatin-bound MCM3 was observed.

These results provide indication that MCM3 is acetylated when the protein is bound to the chromatin, but not when it is free. Moreover, the location of MCM3AP is similar to the acetylated protein migrating in the position of MCM3. MCM3AP is detected only in the structure-bound protein fraction of asynchronized and mimosine treated cells but not nocodazole treated cells. We could not detect MCM3AP in the soluble protein fraction which is consistent with acetylated MCM3 occurring only in the structure-bound protein fraction.

A mutation was generated in the amino acid sequence of MCM3AP, ^{471}HGAG to ^{471}AAAA to confirm that the conserved sequence observed in MCM3AP is important for the acetyltransferase activity of MCM3AP. This mutation is at the center of a motif which is conserved within the GNAT superfamily, which includes N-acetyltransferases and acetyl CoA binding proteins (Dutnall et al (1998)).

His6 tagged wild-type or mutant (^{471}HGAG to ^{471}AAAA)MCM3AP were incubated with or without MCM3 in the presence of ^{14}C labelled acetyl CoA. The same protein amounts of wild type and mutant MCM3AP were examined for their MCM3 acetylase activity with ^{14}C labeled acetyl CoA under the same conditions.

Acetyltransferase activity towards MCM3 was observed to be severely decreased by the mutagenic change. These results confirm that MCM3 is acetylated by MCM3AP and show that the conserved sequence in MCM3AP is important for its acetyltransferase activity.

Y160 yeast strains were transfected with plasmids expressing His6 tagged wild type MCM3AP fused to the Gal4 binding domain and mutant (^{471}HGAG to ^{471}AAAA)MCM3AP fused to the Gal4 activation domain. Cells were cultured for 4 days at 30°C on -LWH SD plates containing 25mM 3-AT.

The mutant MCM3AP was still observed to bind to MCM3. In the presence of MCM3-Gal4 DNA binding domain, the fusion of mutant MCM3AP with the Gal4 activation domain activated the expression of a reporter gene HIS3, as well as the wild type MCM3AP fusion.

The possibility that the acetylation activity affects DNA replication was assessed since the MCM3 is an essential factor for DNA replication.

GFP tagged wild type or mutant (^{471}HGAG to ^{471}AAAA)MCM3AP were expressed in 293T cells. The proportion of GFP positive cells that were also BrdU positive is shown in Figure 3. TOTO3 positive images were used to identify non-transfected cells. The extent of BrdU positive cells in TOTO3 positive but GFP

negative images is indicated as non-transfected.

Expression of wild type MCM3AP decreased the number of DNA-replicating cells whereas expression of mutant MCM3AP showed no decrease compared to cells transfected with vector alone (Figure 3).

When mutant MCM3AP or vector alone were transfected, 35% of cells were labeled with 5-bromo-2'-deoxyuridine (BrdU), whereas, when wild type MCM3AP was transfected, ~20% of cells were labeled. These results demonstrate that the acetylase-activity of MCM3AP can inhibit DNA replication.

MCM3AP was also observed to inhibit DNA replication *in vitro*. 293T cells were transfected with vectors expressing wild type MCM3AP or the acetylase defective MCM3AP mutant described above. Cytosol prepared from both transfectants. The cytosols were incubated with mimosine treated HeLa S3 cell nuclei under the conditions of *in vitro* DNA replication assay.

Cytosol prepared from the MCM3AP transfected cells provided only weak support for DNA replication while cytosol prepared from the cells transfected with the acetylase defective MCM3AP mutant strongly supported replication (see Figure 2).

The present study provides indication that MCM3AP is localized in the nucleus in an MCM3 dependent manner. MCM3AP is imported into the nucleus together with MCM proteins which have an NLS in their amino acid sequence namely MCM2 and MCM3. It is believed that other MCM proteins are imported into the nucleus by binding to these NLS containing MCM proteins [Kimura, 1996][Pasion, 1999].

Indirect immunofluorescence microscopy revealed that

endogenous MCM3AP is a nuclear protein with a diffuse localization comparable to the localization of MCM proteins in G1 phase [Todorov, 1994]. These results show that the binding of MCM3AP to MCM3 occurs in the cytoplasm and persists in the nucleus.

This work has further shown that MCM3AP possesses intrinsic acetyltransferase activity. MCM3AP acetylates both histones and a non-histone protein, MCM3. MCM3 may be a preferred substrate compared to histones, since the addition of MCM3 inhibited the acetylation of histones by MCM3AP and the acetylation of histones by MCM3AP could not be detected under conditions which acetylated MCM3. The work also provides indication that MCM3 is acetylated by MCM3AP *in vivo* as well as *in vitro*, but only when bound to chromatin.

Cell replication is shown to be reduced both *in vitro* and *in vivo* by the acetylase activity of MCM3AP. This demonstrates that the acetylation of MCM3 and/or histone 3 plays an important role in the modulation of cellular proliferation.

Without in any way being bound by theory, a model for the cycle of MCM3 modification is shown in figure 1.

In G1 phase, newly synthesized MCM3AP is bound to MCM3 in the cytoplasm, triggering the nuclear localization of MCM3AP. MCM3 binds to chromatin as a member of pre-replication complex and is acetylated.

In S phase, MCM3 is phosphorylated and is dissociated from chromatin [Fujita, 1998][Schulte, 1995]. The dissociated MCM3 is hyperphosphorylated but not acetylated.

In G2/M phase, all of the MCM3 is dissociated from chromatin

and MCM3AP disappears. MCM3AP may be digested since we could not detect MCM3AP in nocodazole treated cells.

This acetylation cycle indicates that the acetylation of MCM3 is related to the chromatin binding of MCM3, or to MCM3 function while it is chromatin bound.

It has been previously reported that MCM3AP stimulates the nuclear localization of MCM3 and that mutagenesis of amino acids 665K, 666K, 690K, and 700R, in the Lys-rich region of MCM3 affects both the binding of MCM3AP and the nuclear localization of MCM3 [Takei, 1998]. The acetylation of MCM3 by MCM3AP may activate or stimulate binding to Cdc6-bound chromatin and induce the formation of the pre-replication complex.

Alternatively, since we could not conclude that all the chromatin-bound MCM3 is acetylated, the acetylation of MCM3 may result in particular interaction with the chromatin. The initiation of transcription was induced by the binding of MCM3AP to a promoter, indicating that the binding of MCM3AP to chromatin is efficient in remodelling chromatin structure and activating transcriptional complexes. Acetylated MCM3 may set MCM3AP to a chromatin site and induce chromatin remodelling which promotes initiation of DNA replication or RNA transcription. Histone acetylation by MCM3AP may be required in this chromatin remodelling.

In summary, the present inventors have shown that MCM3AP is a nuclear protein which is imported into the nucleus in an MCM3 dependent manner and that MCM3AP is a novel acetyltransferase. MCM3AP acetylates histones and especially MCM3. The experiments provide indication that MCM3AP is involved in the acetylation of MCM3 *in vivo* since acetylated MCM3 was shown

only in the structure-bound fraction and MCM3AP was also fractionated into the structure bound protein fraction.

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CLAIMS:

1. A method for obtaining an agent which modulates the acetylase activity of Mini Chromosome Maintenance 3 Associated Protein (MCM3AP) including:
(a) bringing a MCM3AP polypeptide into contact with a test compound; and
(b) determining the acetylase activity of said MCM3AP polypeptide.

2. A method according to claim 1 comprising determining the acetylation of a Mini Chromosome Maintenance 3(MCM3) polypeptide.

3. A method according to claim 1 comprising determining the acetylation of a histone H3 polypeptide.

4. An assay method for an agent which modulates acetylation of MCM3 by MCM3AP, the method comprising:
(a) bringing into contact a MCM3AP polypeptide, an MCM3 polypeptide and a test compound; and,
(b) determining acetylation of said MCM3 polypeptide.

5. An assay method for an agent which modulates the interaction between MCM3AP and histone H3 including;
(a) bringing into contact a MCM3AP polypeptide, a histone H3 polypeptide and a test compound; and,
(b) determining interaction between said MCM3AP polypeptide and said histone H3 polypeptide.

6. A method according to claim 5 comprising determining the acetylation of said histone H3 polypeptide.

7. A method according to any one of the preceding claims including determining the ability of said test compound to inhibit cellular proliferation.

8. A method according to any one of the preceding claims comprising isolating and/or purifying said test compound.

9. A method according to any one of the preceding claims comprising formulating said agent into a composition which includes one or more additional components.

10. A peptide fragment of MCM3AP polypeptide which modulates interaction between a MCM3AP polypeptide and histone H3.

11. A peptide fragment according to claim 10 which modulates acetylation of histone H3 by MCM3AP.

12. A peptide fragment of a MCM3AP polypeptide which modulates acetylation of MCM3 by MCM3AP.

13. A polypeptide fragment of MCM3AP which acetylates MCM3 or histone H3.

14. An isolated nucleic acid encoding a peptide or polypeptide fragment according to any of claims 10 to 13.

15. An expression vector comprising a nucleic acid according to claim 14 operably linked to regulatory sequences for expression of said peptide or polypeptide fragment.

16. A host cell transformed with an expression vector according to claim 15.

17. A method of making a polypeptide, the method including

culturing a host cell according to claim 16 under conditions for expression of said peptide or polypeptide fragment, and isolating or purifying said peptide or polypeptide fragment.

18. A method according to claim 17 wherein the isolated or purified polypeptide is formulated into a composition comprising one or more additional components.

19. A method of producing an MCM3AP polypeptide comprising; expressing said polypeptide from encoding nucleic acid; and, determining acetylase activity of said polypeptide.

20. A mimetic of a peptide fragment according to any one of claims 10 to 12.

21. An agent obtained by an assay method according to any one of claims 1 to 9.

22. A pharmaceutical composition comprising an agent according to claim 21.

23. Use of a composition according to claim 22 in the manufacture of a medicament for administration for treatment of a disorder of cellular proliferation.

24. Use according to claim 23 wherein the composition comprises a peptide fragment of a MCM3AP polypeptide, a histone H3 polypeptide, a MCM3 polypeptide or a mimetic thereof.

25. A method of making a pharmaceutical composition comprising admixing an agent according to claim 21 with a pharmaceutically acceptable excipient, vehicle or carrier.

26. A method comprising administration of a composition according to claim 22 to a patient for treatment of a disorder of cellular proliferation.

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Figure 1

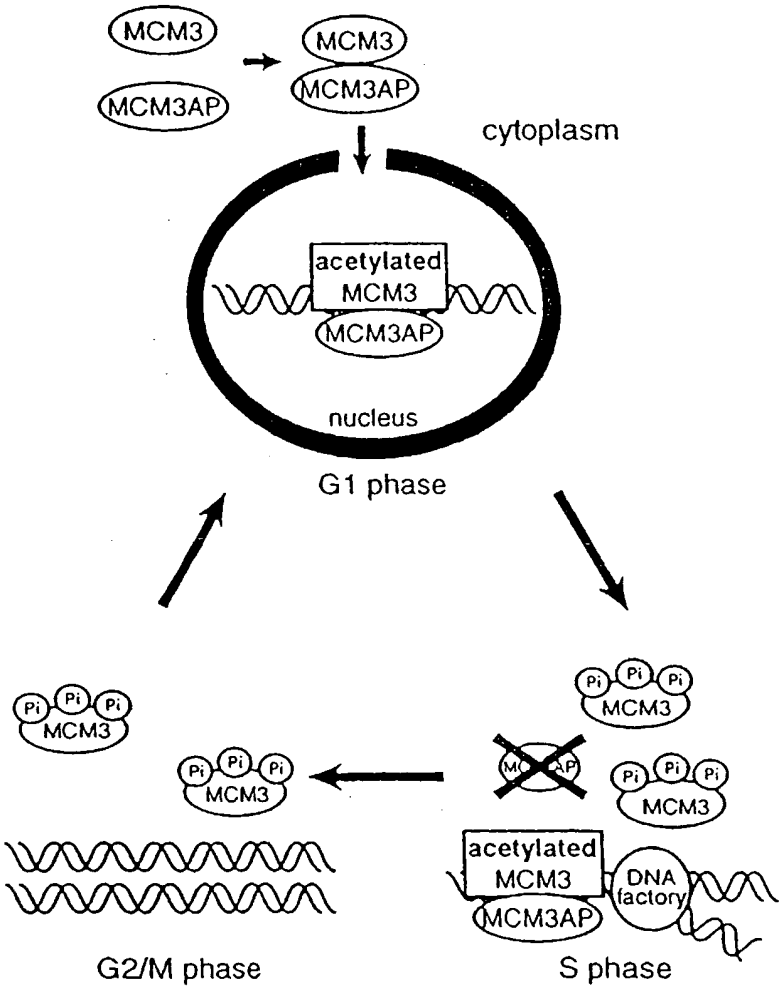


Figure 2

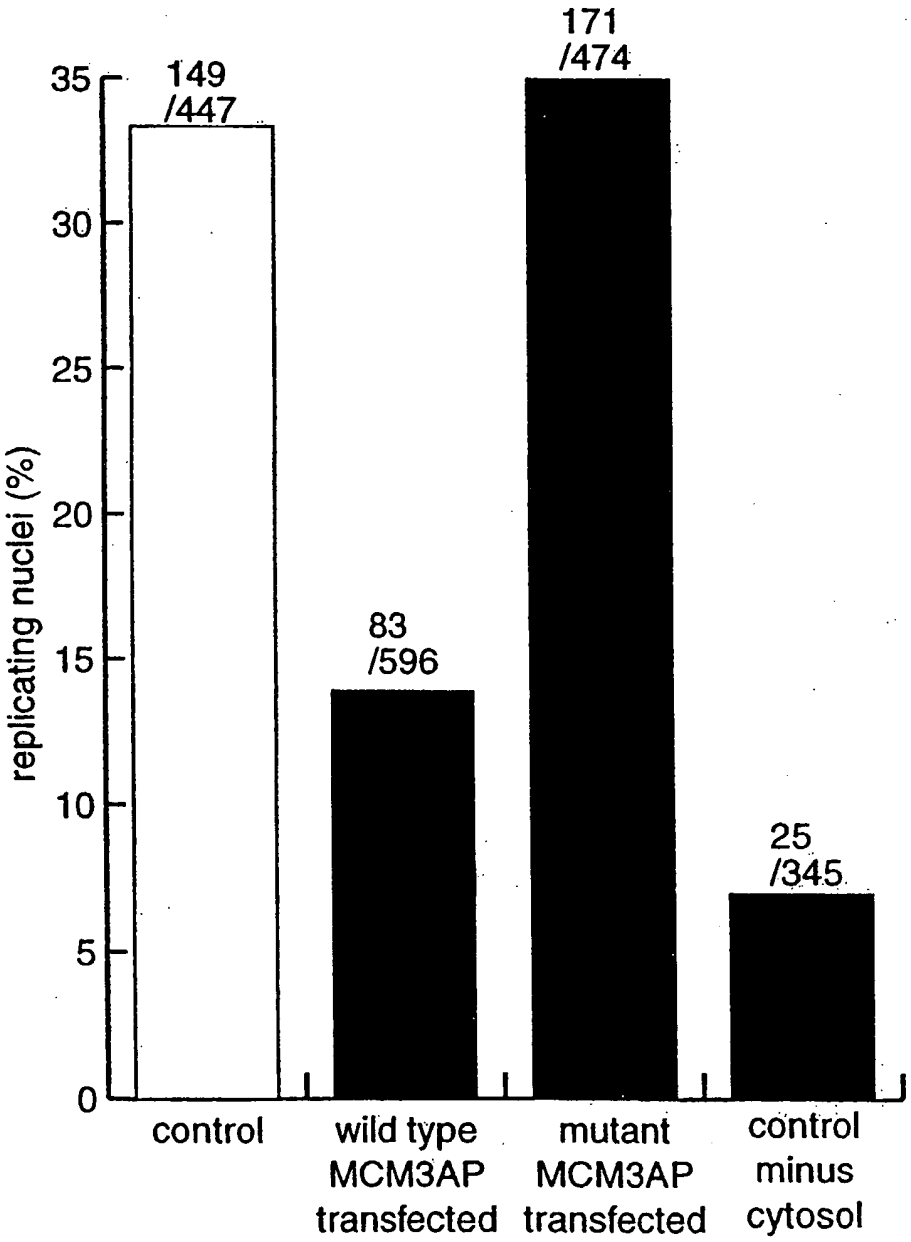
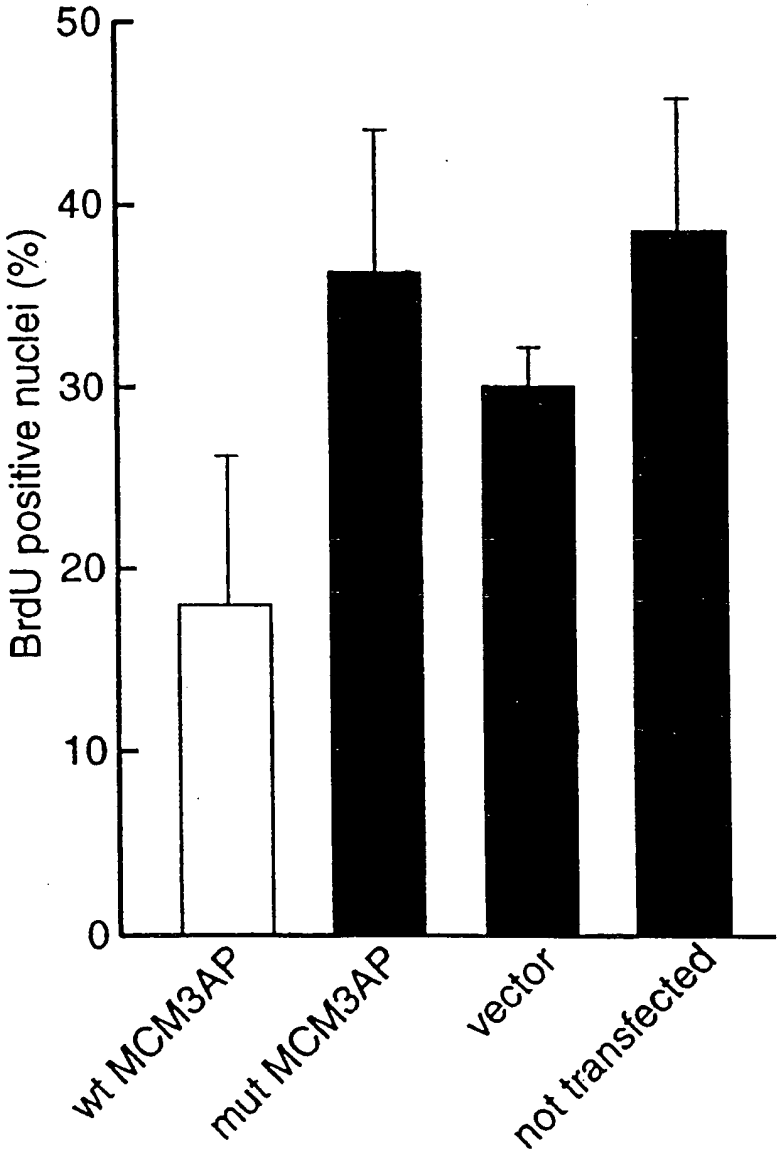

replicating nuclei (%)
35
30
25
20
15
10
5
0
149
/447
83
/596
171
/474
25
/345
control
wild type MCM3AP transfected
mutant MCM3AP transfected
control minus cytosol

Figure 3



INTERNATIONAL SEARCH REPORT

I ional Application No	PCT/GB 01/01892

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12Q1/48 C12N9/10 C12N1/21 C12N1/19
C12N5/10 A61K38/45

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	TAKEI Y ET AL: "MCM3AP, a novel acetyltransferase that acetylates replication protein MCM3" EMBO REPORTS, vol. 2, no. 2, February 2001 (2001-02), pages 119-123, XP001024516 the whole document --- -/--	1,2,4, 7-9,12, 14-21

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 September 2001	09/10/2001

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAKEI Y ET AL: "IDENTIFICATION OF A NOVEL MCM3-ASSOCIATED PROTEIN THAT FACILITATES MCM3 NUCLEAR LOCALIZATION" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 273, no. 35, 28 August 1998 (1998-08-28), pages 22177-22180, XP000941326 ISSN: 0021-9258 cited in the application the whole document ---	
A	KUWAHARA KAZUHIKO ET AL: "A novel nuclear phosphoprotein, GANP, is up-regulated in centrocytes of the germinal center and associated with MCM3, a protein essential for DNA replication." BLOOD, vol. 95, no. 7, 1 April 2000 (2000-04-01), pages 2321-2328, XP002177910 ISSN: 0006-4971 the whole document -----	

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